Technical Tips Online, Vol. 3, 1998

Extension of a cold-labelled oligoprobe for the analysis of PCR products

Rusla M. Dubreuil Lastrucci, Wendy S. Stevens and Barry V. Mendelow

Department of Haematology, School of Pathology, University of the Witwatersrand and South African Institute for Medical Research, 7 York Road, Parktown 2193, South Africa

Keywords: Polymerase chain reaction, Electrophoresis

▼DNA fragment size is often not sufficient to confirm the legitimacy of a PCR product, especially when there is no expectation of specific size but rather of a size range. Further manipulation of the PCR product, such as nuclease restriction or secondary PCR, can be used to confirm that the observed PCR product is real. One approach to the definitive analysis of PCR products is the traditional 'Hot Blot' described by Parker *et al.* (Ref. 1). In this study we describe a new method which achieves the same objective, but without the use of radioactive probes. DNA was extracted from patients with follicular lymphoma, as an example, and PCR was performed using primers designed to amplify the t(14;18) translocation characteristic of this disease (Ref. 2). An oligonucleotide probe for the major breakpoint cluster (mbr) region of *bcl2* was end-labelled with biotin and used to probe the PCR product. The product was run on an electrophoretic gel, electroblotted onto a nylon membrane and detected using streptavidin alkaline phosphatase. A positive result was interpreted if the oligonucleotide probe was able to bind internally to the PCR product and was extended by the Taq polymerase, thus incorporating the cold label into the PCR product. It was found that this method was able to successfully distinguish between legitimate and illegitimate product bands.

Protocol

Template isolation

DNA was extracted from paraffin embedded tissue, blood or marrow of patients with Follicular lymphoma and normal controls according to standard methods (Ref. 3, 4). The oligonucleotides used are described in (Table 1).

Table 1. The primers used in this study spanned the t(14;18) translocation and an amplicon size of 80–300 bp was expected (Ref. 2).

Primer	Sequence
JHa - JH consensus sequence	5′ acc tga gga gac ggt gac c 3′
mbr - major breakpoint region	5′ gag ttg ctt tac gtg gcc tg 3′
Probe - *bcl2* homologous	5′ biotin-gcc tgt ttc aac aca gac c 3′

PCR procedure

A 35-cycle amplification was carried out at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, followed by a final elongation at 72°C for 10 minutes using a PCR core kit (Boehringer Mannheim), according to the manufacturer's instructions.

Standard product analysis

Amplification products were electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualised on a UV transilluminator.

Oligoprobe extension (Ref. 1)

Fifteen microliters of PCR product was added to 50–100 ng of labelled oligonucleotide probe, together with an additional 0.5 μl buffer/$MgCl_2$, 0.3 units Taq DNA polymerase and 0.6 μl of dNTPs, in 20 μl total volume. This mixture was carried through 1 or 2 PCR cycles of 98°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes.

The appropriate known positive and negative controls, as well as a reagent blank, were incorporated in each PCR run. The products of the extension reaction were electrophoresed as before. A molecular-weight ladder [molecular weight marker XIV (Boehringer Mannheim)] was run on

Corresponding author: 075rlast@chiron.wits.ac.za

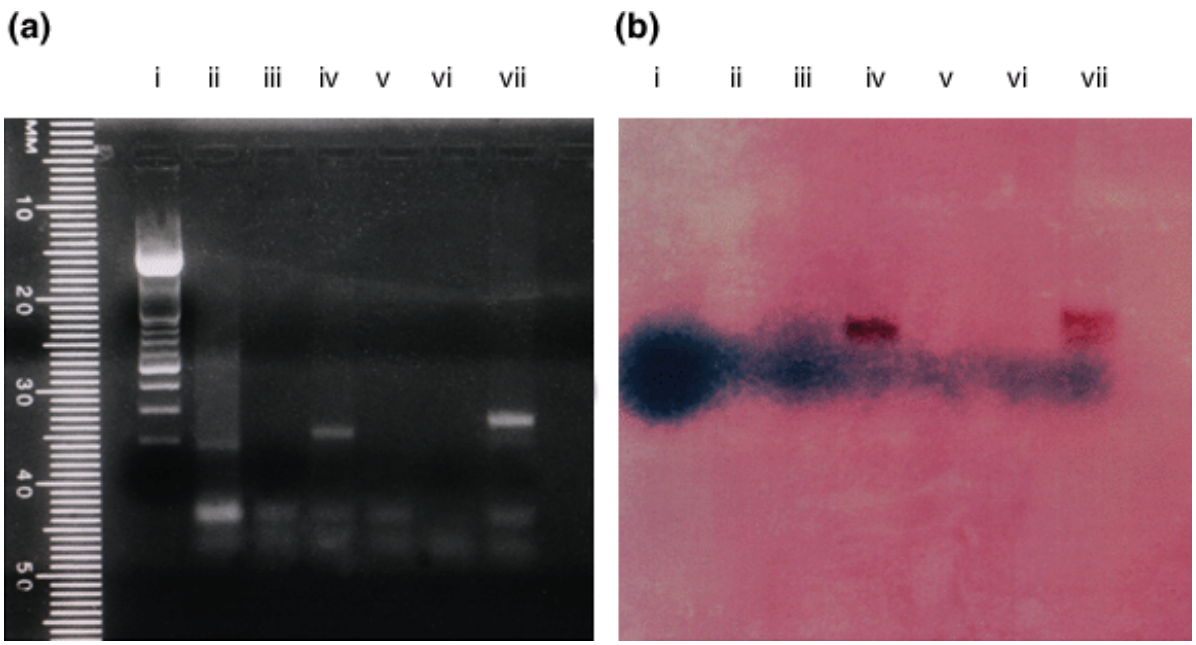

FIGURE 1. Agarose gel (a) and oligonucleotide extension blot (b). (i) Molecular weight ladder, (ii) marrow infiltrated by diffuse large cell lymphoma, (iii) marrow-derived $CD5^+CD19^+$ cells (probable mantle-cell lymphoma), (iv) lymph-node tissue derived from a patient with lymphadenopathy of undetermined aetiology, (v) normal DNA, (vi) reagent blank, (vii) characteristic follicular lymphoma. As a result of this analysis, lane (iv) was interpreted as follicular lymphoma.

each gel to enable accurate size determination. The gel was then visualized, its dimensions measured and transferred onto a positively charged nylon membrane (Boehringer Mannheim), using a Trans-Blot Semi-dry transfer cell (Bio-Rad), or conventional Southern blotting (Ref. 5). The blotted gel and the membrane were checked on the UV transilluminator for efficient transfer and the membrane was air-dried and fixed by exposure to UV for 45 seconds.

Immunological detection of extended oligonucleotide probe

Standard protocols enclosed in the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) were followed, except for the replacement of anti-digoxigenin Fab-fragments conjugated with alkaline phosphatase by streptavidin alkaline phosphatase conjugates (Boehringer Mannheim), where appropriate. The nylon membrane was washed, blocked and incubated with the appropriate alkaline phosphatase conjugate. The membrane was again washed and subsequently incubated with NBT/BCIP, producing an enzyme-catalyzed, insoluble, coloured precipitate.

The oligonucleotide extension method showed a high degree of specificity, as certain bands failed to produce a result post extension. The agarose gel illustrated in (Fig. 1) shows an example of a positive reaction in a characteristic follicular lymphoma, with negative reactions in other lymphomas and the negative control.

Experiments conducted to optimize the oligonucleotide extension protocol (agarose gel and blots not shown) demonstrated the following. (a) Comparison of the two different annealing temperatures, of 43°C and 55°C, during the oligonucleotide extension, showed no difference. The latter temperature was subsequently used as it would afford a higher specificity. The sensitivity of the technique proved to be good, as faint positive bands on PCR became more clearly positive after oligonucleotide extension. (b) The use of two oligonucleotide extension cycles, instead of one, showed no apparent increase in signal. Any more than two cycles is unadvisable because of the possible incorporation of non-specificity at this stage. (c) The use of capillary blotting, instead of electroblotting, showed no significant difference in signal on the nylon membrane. (d) Although the method required the addition of more Taq, nucleotides and buffer, we tried to ascertain whether it would still be possible to carry out the oligonucleotide extension, relying on residual reagents present from the amplification, if only the labelled oligoprobe was added. It was found that although oligonucleotide extension did occur, it was more efficient when oligonucleotide extension was performed sooner after amplification. Optimal oligonucleotide extension was, however, still obtained when fresh reagents were added.

This technique allowed oligonucleotide extension and detection, without the use of radioactivity, quickly and

easily. Ample labelled probe was obtainable. This could be stored indefinitely, without requiring repeated and expensive probe labelling. The method required that the molecules be transferred to a gel before detection; transfer of the attached digoxigenin and biotin moieties appeared to present no problem, and was quickly and easily achieved. The method still has the advantage of fragment size as a cross check, unlike some of the techniques that rely exclusively on the presence of a signal.

In short, this method was found to successfully and repeatedly distinguish between legitimate and illegitimate PCR product bands, in a simple, safe and easily executable manner, rendering PCR results reliable. Studies are under way to further explore the specificity of this technique for variants of the t(14;18) and various other translocations. Table 1.

References

1 Parker, J.D. and Burmer, G.C. (1991) *Biotechniques* 10, 94–101.
2 Segal, G.H., Jorgensen, T., Scott, M. and Braylan, R.C. (1994) *Hum. Pathol.* 25, 1276–1282.
3 Stevens, A. (1982) Bancroft, J.D. and Stevens, A. (Eds.). Theory and Practice of Histological Techniques, 2nd edition, Churchill and Livingston 113.
4 Ausubel, F.M. *et al.* (1996) Current Protocols in Molecular Biology, John Wiley & Sons, Inc. 2.2.1–2.2.3.
5 Fitsch, E.F., Sambrook, J. and Maniatis, T. (1989) Molecular Cloning, a Laboratory Manual, 2nd edition, Cold Spring Harbour Laboratory Press 9.34–9.37.

Products Used

PCR core kit: PCR core kit from Boehringer Mannheim
molecular weight marker XIV: molecular weight marker XIV from Boehringer Mannheim
nylon membrane: nylon membrane from Amersham Pharmacia Biotech
Trans-Blot Semi-dry transfer cell: Trans-Blot Semi-dry transfer cell from Bio-Rad
DIG Nucleic Acid Detection Kit: DIG Nucleic Acid Detection Kit from Boehringer Mannheim
Alkaline Phosphatase: Alkaline Phosphatase from Boehringer Mannheim
alkaline phosphatase: alkaline phosphatase from Tropix Inc